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Mechanism of action and resistant profile of anti-HIV-1 coumarin derivatives

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Abstract

Dicamphanoyl khellactone (DCK) is a coumarin derivative that can potently inhibit HIV-1 replication. DCK does not inhibit RNA-dependent DNA synthesis. However, an HIV reverse transcriptase (RT) inhibitor-resistant strain, HIV-1/RTMDR1, is resistant to DCK. Thus, it is possible that HIV-1 RT is the target of DCK. To test this possibility, DCK-resistant viruses were selected in the presence of DCK. Our results indicate that a single amino acid mutation, E138K in HIV-1 RT, is sufficient to confer DCK resistance. Interestingly, a DCK derivative, 3′ R,4′ R-Di-*O*-(-)-camphanoyl-2-ethyl-2′ ,2′ -dimethyldihydropyrano[2,3-f]chromone (DCP8), is effective against HIV-1/RTMDR1. However, the DCK-escape virus carrying the E138K mutation remains resistant to DCP8. Since DCK did not inhibit the RNA-dependent DNA polymerase activity of HIV-1 RT when using poly-rA or poly-rC as template, we evaluated the effect of DCK on the DNA-dependent DNA polymerase activity of HIV-1 RT. Our results indicate that DCK can inhibit the DNA-dependent DNA polymerase activity of HIV-1 RT. In conclusion, DCK is a unique HIV-1 RT inhibitor that inhibits the DNA-dependent DNA polymerase activity. In contrast, DCK did not significantly affect the RNA-dependent DNA polymerase activity when poly-rA or poly-rC was used as templates. An E138K mutation in the non-nucleoside RT inhibitors (NNRTIs) binding pocket of HIV-1 RT confers resistance to DCK and its chromone derivative, DCP8.

Keywords: Coumarin; Polymerase activity; Mutation

Introduction

Since the AIDS pandemic began in the early 1980s, many efforts have been made to search for effective anti-HIV-1 drugs. As a result, numerous compounds were found to have anti-HIV activity. Many of the anti-HIV-1 agents targeting HIV-1 reverse transcriptase, protease, and gp41 have been used for AIDS therapy. Highly active antiretroviral therapy (HAART), which combines both HIV-1 reverse transcriptase and protease inhibitors, has been effective in controlling the viral load in HIV-1-infected individuals. Although HAART can effectively control plasma viremia, the virus is suppressed rather than eradicated (Chun et al., 1997). In addition, several drawbacks, such as the emergence of drug resistance and side effects associated with the antiretroviral agents, compromise many HAART regimens. Therefore, new anti-HIV compounds with unique mechanisms of action are needed to further improve HAART.

DCK is one of the coumarin derivatives that exhibit potent anti-HIV activity (Huang et al., 1994). The chemical structure and mechanism of action of DCK are different from the drugs currently used in AIDS therapy. The structure of DCK is illustrated in Fig. 1. DCK was shown to inhibit HIV-1 at sub-micro molarity concentrations. Due to the potent anti-HIV activity, many DCK derivatives were synthesized and were shown to have improved anti-HIV activity (Xie et al., 1999; Yu et al., 2003). Although DCK is a potent inhibitor for many HIV-1 isolates, including primary isolates, the compound is ineffective against an

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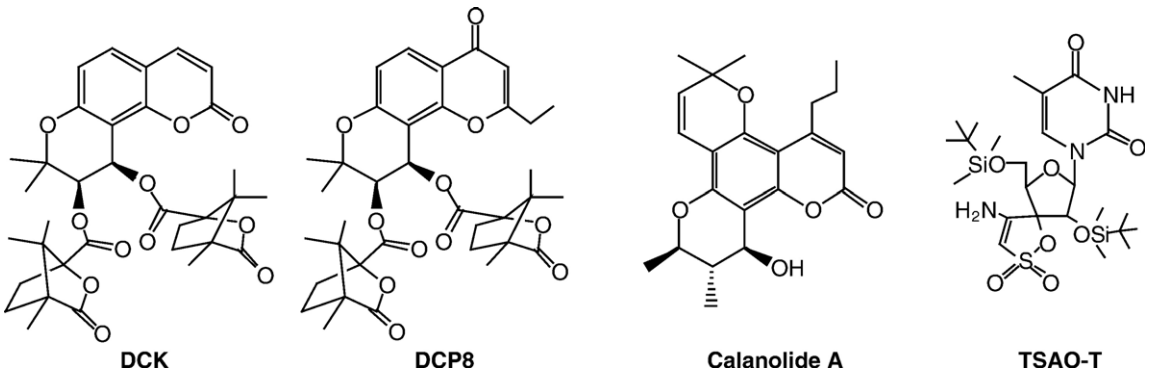

Fig. 1. Chemical structure of DCK, DCP8, Calanolide A, and TSAO-T.

HIV-1 strain resistant to both nucleoside RT inhibitors and NNRTIs (Yu et al., 2004). In order to improve the drug-resistant profile, a series of compounds with structural modifications on the khellactone ring of DCK was synthesized and shown to be effective against the multiple-RT inhibitor-resistant strain (Yu et al., 2004). The resistance of the pre-existing multiple-RT inhibitor-resistant strain to DCK suggests that HIV-1 RT might be the target of the compound. DCK is inactive in an assay that primarily detects the RNA-dependent DNA polymerase activity of HIV-1 RT (Huang et al., 1994). DCK appears to inhibit HIV-1 at a step post-HIV-1 entry, but before integration of HIV-1 DNA into host chromosomes.

In this study, we have evaluated the activity of DCK against HIV-1 strains that are resistant to multiple RT inhibitors and protease inhibitors. We also selected DCK-resistant mutants and analyzed the genotypes of the DCK escape mutants. An E138K mutation in the HIV-1 RT is responsible for the drug resistance. Similar to the reverse transcriptases in other retroviruses, HIV-1 RT possesses RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities. Unlike other RT inhibitors, DCK appears to inhibit the DNA-dependent DNA polymerase activity without affecting the RNA-dependent DNA polymerase activity of HIV-1 reverse transcriptase.

Results

HIV-1 resistant to multiple RT inhibitors is less sensitive to DCK

In an effort to determine whether DCK can inhibit pre-existing drug-resistant strains, we have tested the anti-HIV activity of DCK against HIV-1/RTMDR1 and HIV-1/PRMDR. HIV-1/RTMDR1 contains four mutations, M41L, L74V, V106A, and T215Y, in HIV-1 RT rendering the virus resistant to multiple HIV-1 RT inhibitors, including nucleoside analogs and NNRTIs (Larder et al., 1993). HIV-1/PRMDR, containing mutations M46I, L63P, V82T, and I84V in HIV-1 protease, is resistant to multiple protease inhibitors (Condra et al., 1995). These two drug-resistant strains and HXB-2, a T-cell line adapted HIV-1 molecular clone, were tested for their sensitivity to DCK. HXB-2 is very sensitive to DCK (Fig. 2). The concentration of DCK required to inhibit 50% of HXB-2 replication (IC50) is 90 nM. The sensitivity of HIV-1/PRMDR to DCK is comparable to that of HXB-2. In contrast, HIV-1/RTMDR1 is approximately 2 logs less sensitive to DCK (Fig. 2). Since HIV-1/RTMDR1 is a drug-resistant strain constructed in the genetic background of HXB-2, these results strongly suggest that HIV-1 RT might be the target of DCK.

An E138K mutation in HIV-1 RT is responsible for DCK resistance

HIV-1/RTMDR1 has four mutations at amino acid residues M41L, L74V, V106A, and T215Y of HIV-1 RT that are responsible for its drug resistance to multiple HIV RT inhibitors (Larder et al., 1993). These mutations did not arise from exposure to DCK. In order to gain insight into the interaction between DCK and its target, drug escape mutants were selected by growing NL4-3 virus in the presence of escalating doses of DCK. The drug-resistant mutants were approximately 2 logs less sensitive to DCK. Sequence analysis of the DCK escape variants revealed two mutations, E138K and G550K, in the HIV-1 RT (Fig. 3A). These two mutations were re-introduced into the wild type NL4-3 molecular clones using site-directed mutagenesis and were analyzed for their role in DCK resistance. The two viruses

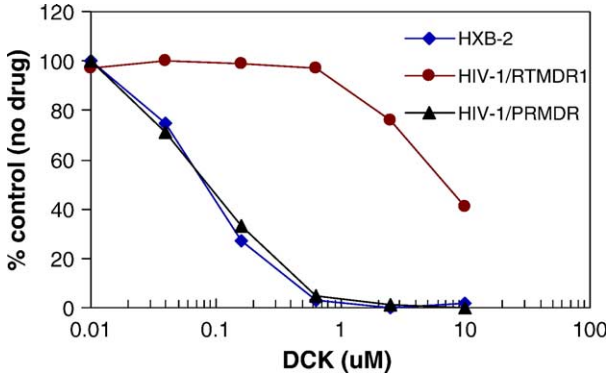

Fig. 2. A multiple HIV-1 RT inhibitor-resistant strain is resistant to DCK.

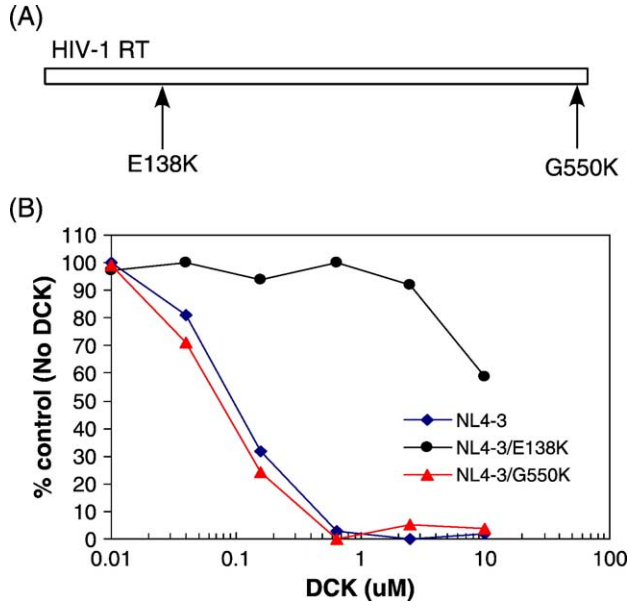

Fig. 3. (A). Changes in amino acid residues in the HIV-1 RT of DCK escape variants. (B). An E138K mutation confers DCK-resistant phenotype.

derived from the mutagenesis are NL4-3/E138K and NL4-3/G550K. The G550K mutation did not significantly affect DCK sensitivity. On the other hand, the E138K mutation was fully responsible for the resistance to DCK (Fig. 3B). The E138K mutation in the RT of NL4-3 resulted in a greater than 100-fold decrease in DCK sensitivity. This result suggests that E138 of HIV-1 RT plays a key role in the anti-HIV activity of DCK.

DCP8 is effective against HIV-1/RTMDR1, but not NL4-3/E138K

DCP8 is one of DCK derivatives designed to improve the drug-resistant profile of DCK (Yu et al., 2004). The major difference between DCK and DCP8 is at the khellactone ring of the molecule (Fig. 1). DCP8 is the most potent chromone derivative against HIV-1/RTMDR1. Although HIV-1/RTMDR1 is resistant to DCK, the virus is sensitive to DCP8 (Fig. 4). The IC50s of DCK and DCP8 against HIV-1/RTMDR1 are 8 and 0.06 μM, respectively. Thus, a structural change on the khellactone ring can overcome the DCK resistance of HIV-1/RTMDR1. However, the DCK-resistant mutant, NL4-3/E138K, remains resistant to DCP8 (Fig. 4). These results strengthen the notion that the amino

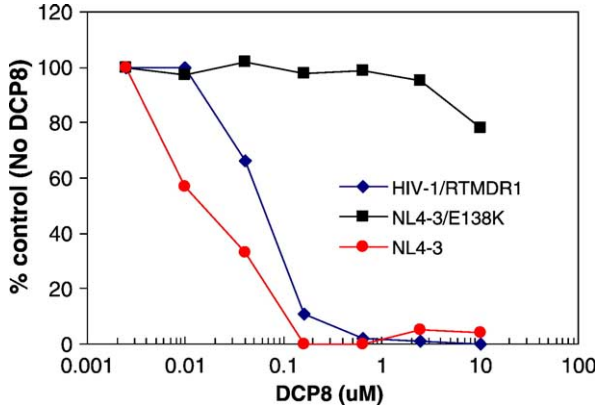

Fig. 4. DCP8 is effective against HIV-1/RTMDR1, but not NL4-3/E138K.

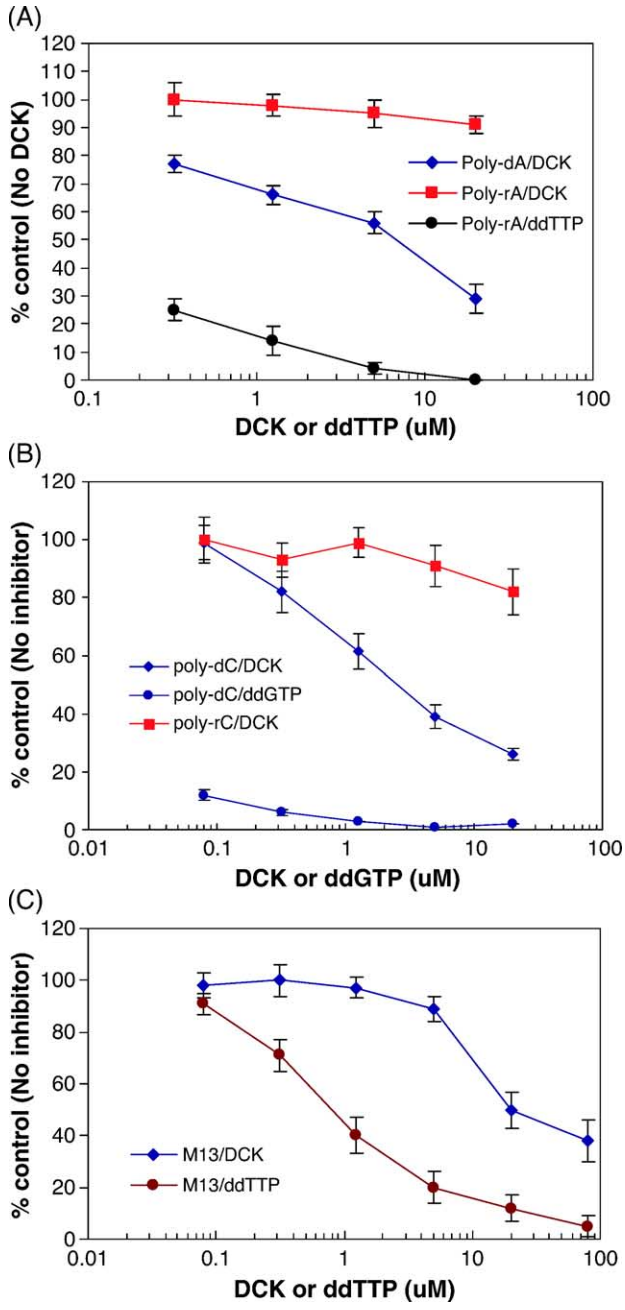

Fig. 5. DCK inhibits the DNA-dependent DNA polymerase activity of HIV-1 RT. NL4-3 lysate in 1% Triton X-100 was used as HIV-1 RT in the assays. (A) Poly-dA (Poly-dA/DCK) was used as templates to replace poly rA (Poly-rA/DCK) in the HIV-1 RT assays to detect the DNA-dependent DNA polymerase activity. (B) Poly-dC (Poly-dC/DCK) was used as templates to replace poly-rC (Poly-rC/DCK) in the HIV-1 RT assays to detect DNA-dependent DNA synthesis, using poly-dC/oligo-dG as template/primer. (C) M13mp18 plus strand DNA was used as templates. The primer used in the assay is described in Materials and methods. Each data point represents the average of two duplicated experiments.

acid residue, E138 in HIV-1 RT, plays a key role in the anti-HIV-1 activity of DCK and DCP8. Further structural changes on DCK or DCP8 are needed to improve the anti-HIV-1 activity against NL4-3/E138K.

DCK inhibits DNA-dependent DNA polymerase activity of HIV-1 RT

We have previously shown that DCK does not inhibit HIV-1 RT in an assay used primarily to detect the RNA-dependent DNA polymerase activity (Huang et al., 1994). However, the drug-resistant studies described above strongly suggest that HIV-1 RT is the target of DCK. This raises the possibility that DCK interacts with HIV-1 RT in a unique way by inhibiting the DNA-dependent DNA polymerase activity without affecting the RNA-dependent DNA polymerase activity of HIV-1 RT. To test this possibility, we used DNA to replace the RNA templates to detect the DNA-dependent DNA polymerase activity of HIV-1 RT. The known DNA chain terminators, ddTTP and ddGTP, were used as positive controls for the HIV-1 RT assay. In agreement with previous report (Huang et al., 1994), DCK did not inhibit HIV-1 RT activity when poly-rA or poly-rC was used as templates (Figs. 5A and B). In contrast, DCK can inhibit the DNA-dependent DNA polymerase activity of HIV-1 RT when poly-dA or poly-dC was used as templates (Figs. 5A and B). Unlike the heteropolymeric nature of provial DNA, poly-dA and poly-dC are homopolymeric templates. To determine whether the inhibitory activity of DCK can be observed using heteropolymeric templates, we used M13mp18 plus strand DNA as templates in the HIV-1 RT assay. Both ddTTP and DCK inhibited the DNA-dependent DNA synthesis of HIV-1 RT when M13mp18 was used as templates (Fig. 5C). The inhibitory activity of DCK and ddTTP is approximately 3-fold and 10-fold, respectively, less potent when compared with assays in which poly-dA or poly-rA was used as templates (Fig. 5A). Although the DCK concentration required to inhibit the DNA-dependent DNA polymerase activity is higher than that required to inhibit HIV-1 replication, the data support the notion that HIV-1 RT is the target of DCK.

Discussion

HIV-1 RT inhibitors, nucleoside analogs, or non-nucleoside analogs are able to inhibit RNA-dependent DNA polymerase activity. DCK appears to be able to inhibit the HIV-1 RT without significant effect on RNA-dependent DNA synthesis when poly-rA or poly-rC was used as templates. The unique mechanism of action might allow DCK, or its derivatives, to complement with other anti-HIV-1 agents.

Among all of the known HIV-1 RT inhibitors, Calanolide A and its derivatives share some structural similarity to DCK (Fig. 1). Like other HIV-1 RT inhibitors, Calanolide A inhibits HIV-1 reverse transcription (Buckheit et al., 1999). Calanolide A-resistant viruses carry T139I, L100I, Y188H, or L187F mutations in the RT (Buckheit et al., 1999). The proximity of T139 and E138 in HIV-1 RT suggests that Calanolide A and DCK binding sites are likely to be in close proximity. The E138K mutation is not unique to the DCK-resistant virus. The identical mutation, E138K, was found to confer drug resistance to the HIV-1 RT inhibitor, [2′,5′-Bis-*O*-(tert-butyldimethylsilyl)- β-D-ribofuranosyl]-3′-spiro-5′-(4'-amino-1',2′-oxathiole-2′,2′-dioxide)thymine (TSAO-T) (Balzarini et al., 1993; Jonckheere et al., 1994). The chemical structure of TSAO-T is illustrated in Fig. 1. There is no obvious structural similarity between DCK and TSAO-T. E138 is one of the amino acid residues located in a well-defined binding pocket for NNRTIs (Esnouf et al., 1997; Kohlstaedt et al., 1992). Although TSAO-T is structurally distinct from both DCK and Calanolide A (Fig. 1), it is possible that the interactive sites of DCK, DCP8, Calanolide A, and TSAO-T are in close proximity. It would be of interest to determine whether there is a cross resistance among these four anti-HIV-1 RT inhibitors.

Both NL4-3/E138K and HIV-1/RTMDR1 are highly resistant to DCK. One of the four mutations, V106A, in the RT of HIV-1/RTMDR1 is responsible for the resistance to multiple NNRTIs (Brenner et al., 2003; Larder, 1992). Similar to E138, V106 is one of the key amino acids in the NNRTI binding pocket of HIV-1 RT. Thus, the NNRTI binding pocket is a key determinant for DCK sensitivity. It is interesting that the DCK derivative, DCP8, is able to overcome the drug resistance derived from the V106A mutation, but not the E138K mutation in the RT. It should be noted that E138 in the NNRTI binding pocket is located in the p51 subunit of the p51/p66 HIV-1 RT heterodimer. Other key amino acids residues, such as V106A, in the NNRTI binding pocket are primarily located in the p66 subunit (Esnouf et al., 1997). Therefore, it is possible that the primary interactive site for DCK and DCP8 is in the p51 subunit of HIV-1 RT.

Why DCK appears to inhibit DNA-dependent DNA polymerase activity without affecting the RNA-dependent DNA polymerase activity? One possibility is that DCK binds to a site essential for the interaction between HIV-1 RT and DNA template but not RNA template. Alternatively, DCK could bind to the p51 subunit and interfere with second strand transfer before (+) DNA strand elongation. It is also possible that DCK impedes the DNA strand displacement at a late stage of viral DNA replication. DNA strand displacement appears to be required for DNA-dependent DNA synthesis during viral replication (Amacker et al., 1995). Due to its unique mode of action, DCK and its derivatives could be used to functionally dissect HIV-1 RT and might have the potential to be clinically useful.

Materials and methods

Viruses

HIV-1/RTMDR1, an HIV-1 mutant resistant to multiple RT inhibitors (Larder et al., 1993), and HIV-1/PRMDR, an HIV-1 strain resistant to multiple protease inhibitors (Condra et al., 1995), were obtained from the NIH AIDS Research and Reference Reagent Program. HXB-2 is a molecular clone derived from HIV_{LAV} (Ratner et al., 1987). NL4-3 is a chimeric molecular clone derived from NY5 and HIV_{LAV} (Adachi et al., 1986).

Selection of DCK-resistant viruses

Selection of DCK-resistant NL4-3 variants was carried out using MT4 cells. The drug-resistant mutants were generated using escalating concentrations of DCK starting at a concentration that inhibits 95% of HIV-1 replication as previously described (Holz-Smith et al., 2001; Yuan et al., 2004). Viral replication was monitored by measuring p24 level in the culture supernatants. The HIV-1 RT gene of the selected DCK-resistant variants was determined using an automated DNA sequencer (Bio-Synthesis, Lewisville, TX).

Anti-HIV assay

A modified HIV-1 infectivity assay previously described was used in the experiments (Zhu et al., 2001). A diluted HIV-1 stock at a multiplicity of infection (MOI) of 0.001 TCID50/cell was used to infect MT4 cells. Twenty microliters of the virus was incubated with 20 μl of compounds in RPMI 1640 that contains 10% fetal bovine serum in a 96-well microtiter plate. Twenty microliters of MT4 cells at 6×10^5 cells/ml were added to each well, and the cell culture was incubated at 37 °C in a humidified CO_2 incubator. Two days after infection, fresh media (180 μl) containing appropriate concentrations of the compounds were added to each well. On day 4 post-infection, supernatant samples were harvested and assayed for p24 using an ELISA kit from ZeptoMetrix Corporation, Buffalo, NY.

HIV RT assay

HIV-1 RT activity was determined by modifying a previously published methods (Goff et al., 1981; Willey et al., 1988). Briefly, HIV-1 NL4-3 culture supernatants were treated with 1% (vol/vol) Triton X-100. A 10 μl sample of each viral lysate was mixed with 50 μl of a reaction cocktail containing 50 mM Tris–HCl (pH 7.8), 75 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 5 μg/ml Poly rA, 1.5 μg/ml Oligo $dT_{12–18}$, 0.1 μM dTTP, 0.05% NP-40, and 10 μCi/ml ^{32}P-TTP. The reaction mixtures were incubated at 37 °C for 90 min. Aliquots (50 μl) of reaction mixtures were spotted onto DE-81 paper (Whatman) in a sample filtration manifold (Schleicher and Schuell) and washed three times with 2 × SSC (0.3M NaCl, 0.03M NaCitrate). Radioactivity was quantified with a Packard Matrix 9600 Direct Beta Counter. The reaction cocktail was modified when M13mp18 plus strand DNA (Sigma-Aldrich, St. Louis, MO) was used as template. In addition to the template, an oligonucleotide (5′ GGCCAGTGCCAAGC-3′) complementary to the immediate down stream of the polylinker region of M13mp18 and dNTPs (a mixture of four nucleotides) were used to replace Oligo $dT_{12–18}$ and dTTP, respectively.

Mutagenesis

A quick exchange DNA mutagenesis procedure was used to introduce desired mutations at specific sites (Holz-Smith et al., 2001; Yuan et al., 2004). A pair of primers, 5′ -GTA TAA ACA ATA AGA CAC CAG GG-3′ and 5′ -CCC TGG TGT CTT ATT GTT TAT AC-3′ , were used to introduce the E138K mutation into NL4-3 virus. The detailed protocol is described in the brochure provided by the manufacturer, Strategene. The sequences of the desired mutants were confirmed by DNA sequencing.

Acknowledgments

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